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We present a scalable synthesis of 3'-amino-3'-deoxy-2-thio-thymidine-5'-phosphoro-2-methylimidazolide, an activated monomer that can copy adenosine residues in nucleic acid templates rapidly without a polymerase. The sulfur atom substitution enhances the rate of template copying by 5-fold compared with the 3'-amino-3'-deoxy-T monomer, while the 3'-amino monomers exhibit a 2- to 30-fold enhancement compared with their ribonucleotide counterparts.

Nonenzymatic template-directed replication of nucleic acids has been hypothesized to be the mechanism of information transfer in primitive cells prior to the advent of ribozyme polymerases.<sup>1</sup> Early efforts involving high-energy nucleotide monomers such as 5'-phosphoro-2-methylimidazolides (or 2-MeImpNs) (Fig. 1, top) showed that RNA templates consisting of C residues can be copied by 2-MeImpG in hours to days in the presence of divalent cations (typically  $Mg^{2+}$ ).<sup>2</sup> However, no enzyme-free process has yet been discovered that enables the rapid and efficient copying of mixed-sequence RNA templates with activated ribonucleotide monomers. This problem has stimulated interest in alternative nucleic acids that might exhibit faster replication chemistry; such polymers are of interest both with respect to the origin of life and in the context of designing artificial life forms based on non-biological chemistry. The most promising non-biological nucleic acids are the phosphoramidate polymers, which are assembled from nucleotides with an amino group on the sugar instead of the less nucleophilic hydroxyl. N3'-P5'-linked phosphoramidate DNA<sup>3,4</sup> (3'-NP-DNA, Fig. 1, bottom) stands out as an

## Synthesis of activated 3'-amino-3'-deoxy-2-thio-thymidine, a superior substrate for the nonenzymatic copying of nucleic acid templates†

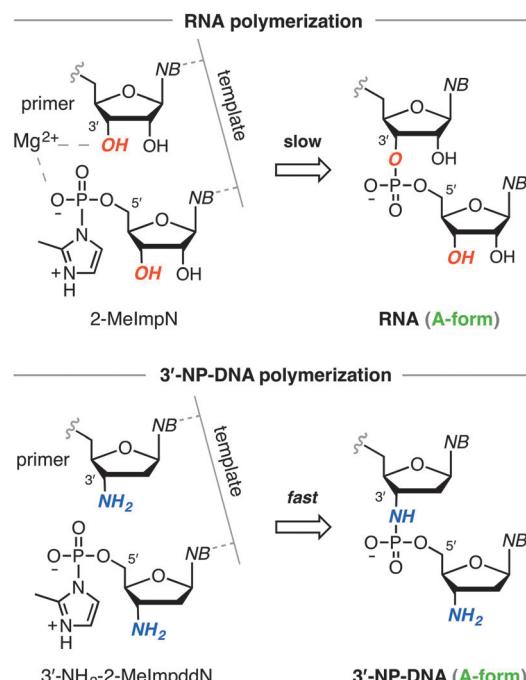
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Fig. 1 Template-directed polymerization of RNA (top) and 3'-NP-DNA (bottom). NB denotes nucleobase.

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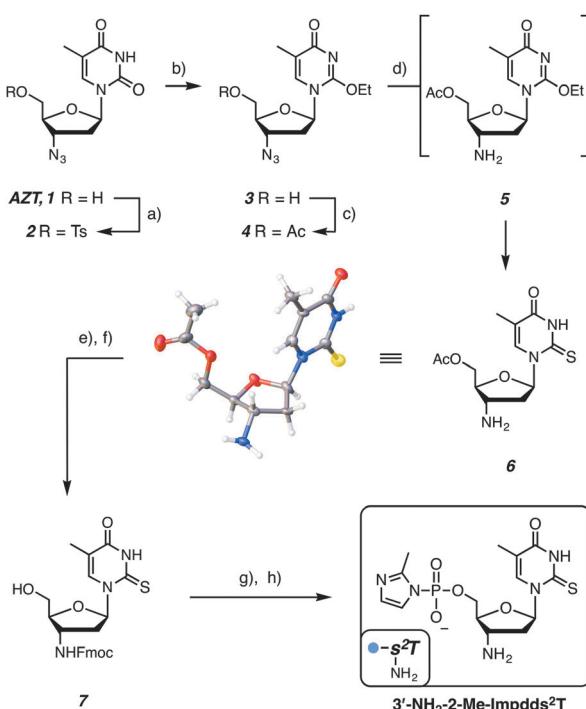
attractive alternative genetic model because it adopts a helical geometry that is similar to that of A-form RNA.<sup>5</sup> A-form geometry is the preferred conformation for the nonenzymatic template-directed oligomerization of activated ribonucleotides<sup>6,7</sup> most likely because the A-form double helix of RNA brings the 3'-OH group of the primer in line with the leaving group of the incoming monomer. We have previously shown<sup>8</sup> that activated 3'-amino-2',3'-dideoxynucleotide monophosphates (3'-NH<sub>2</sub>-2-MeImpddNs) (Fig. 1, bottom) rapidly polymerize on short, homopolymeric DNA, RNA, and locked nucleic acid (LNA) templates. We also found<sup>9</sup> that replacing 3'-amino-T with 3'-amino-2-thio-T enhances the rate and fidelity of 3'-NP-DNA synthesis in the copying of DNA, RNA and 3'-NP-DNA templates. However, further progress in the



development and quantitative analysis of this system has been hindered by lack of access to the critical substrate, the 5'-phosphoro-imidazolide of 3'-amino-2-thio-T (3'-NH<sub>2</sub>-2-MeImp-dds<sup>2</sup>T), which could previously be synthesized only in small quantities because of the expensive starting material and the very low yield of the desired product.

Here, we present a concise and scalable synthesis of 3'-amino-2-thio-T and the corresponding 5'-phosphoro-imidazolide, 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T. We also present the first quantitative investigation of template-copying kinetics using this activated nucleotide. Our results show that nonenzymatic 3'-NP-DNA synthesis using the 2-thio modified 3'-amino-T phosphoro-imidazolide monomer is significantly faster than with unmodified 3'-amino-T, and is also considerably faster than RNA synthesis with activated U and 2-thio-U.

Our earlier attempts to establish a concise synthetic route to 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T involved regioselective thio-carbonylation<sup>10</sup> of 4-O-protected 3'-azido-3'-deoxy-thymidine. However, these substrates afforded only deglycosylation products using Lawesson's reagent, an observation later reported<sup>11</sup> for 4-O-mesityl-3',5'-O-di(TBS)-thymidine. We then studied nucleophilic ring opening of 2-thio-2,3'-cyclonucleosides by azides at the 3'-position, but observed little to no conversion (by <sup>1</sup>H NMR analysis) of the substrates in the presence of either excess LiN<sub>3</sub> or TMSN<sub>3</sub> accompanied by various Lewis acids [*e.g.*, Hg(OAc)<sub>2</sub>, Er(OTf)<sub>3</sub> and Yb(O-iPr)<sub>3</sub>].

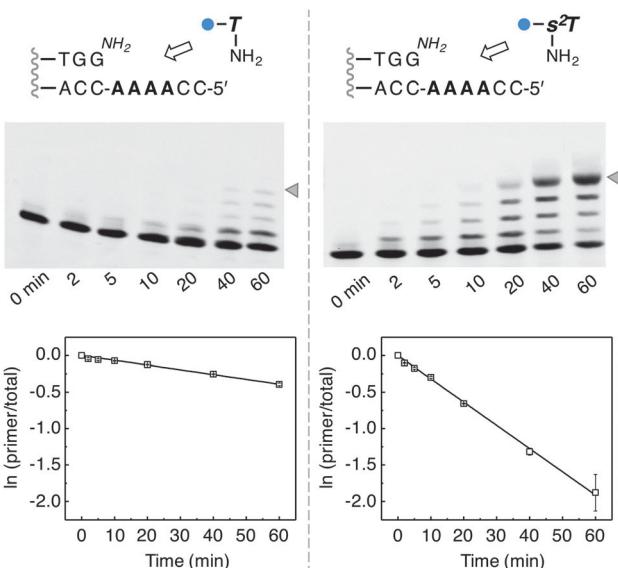


**Scheme 1** Synthesis of 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T. Reaction conditions: (a) TsCl, pyr, 0 to 20 °C, 6 h, 73%; (b) DBU, EtOH, 85 °C, 24 h, 81%; (c) DMAP (cat.), Ac<sub>2</sub>O, 20 °C, 12 h, >95%; (d) H<sub>2</sub>S (gas), TMG, pyr, 0 to 20 °C, 16 h, 53%; (e) 7N NH<sub>3</sub> in MeOH, 20 °C, 92%; (f) Fmoc-OSu, Na<sub>2</sub>CO<sub>3</sub> (aq), pyr, 0 to 20 °C, 6 h, 78%; (g) 1. POCl<sub>3</sub>, 2,6-lutidine, PO(OMe)<sub>3</sub>, 3 Å MS, 0 to 20 °C, 2 h; 2. 2-Me-imidazole, 2 h, 30%; (h) piperidine, DMF, 0 °C, 0.5 h, 85%. X-ray structure: H: white, C: gray, O: red, N: blue, and S: yellow.

Our successful strategy to access 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T (Scheme 1) commenced with the 5'-tosylation<sup>12</sup> of 3'-azido-3'-deoxythymidine (AZT) 1.<sup>13</sup> Intramolecular displacement of the 5'-tosylate by the C2-oxyanion formed in the presence of a Brønsted base (*e.g.*, DBU) yielded the 2,5'-O-anhydro-cyclonucleoside, and subsequent ring-opening in refluxing ethanol afforded the 2-thiothymidine 3 in 59% overall yield from 1. After converting 3 into the acetate 4, we were able to incorporate the sulfur atom into the nucleobase using H<sub>2</sub>S in the presence of tetramethylguanidine (TMG)<sup>14</sup> (see Fig. S1 in the ESI† for details of the reaction setup). <sup>1</sup>H NMR analysis of an aliquot of the crude reaction mixture after 1 hour revealed that 4 was fully consumed, while two new species were formed: 3'-amino-2-ethoxythymidine 5 and 3'-amino-2-thio-thymidine 6, in a molar ratio of 2:1 (5/6). The relative abundance of 6 continued to increase as the reaction progressed [up to *ca.* 1:3 (5/6) after 6 hours]. We did not observe (by either ESI or <sup>1</sup>H NMR analysis) the formation of any 3'-azido-2-thio-thymidine, suggesting that the incorporation of sulfur was slower than the reduction of the 3'-azide, and that 6 was likely formed from 5. The structure of the 2-thio nucleoside 6 was confirmed by both <sup>1</sup>H-<sup>1</sup>H gCOSY NMR spectroscopy and X-ray crystallography (see the ESI†). We then converted 6 into the phosphoroimidazolide precursor 7 *via* 5'-deacetylation and Fmoc protection of the 3'-amine. A one-flask 5'-O-phosphorylation and 2-methyl-imidazolide synthesis, followed by the removal of Fmoc, provided 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T in 26% overall yield from 7.

With 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T in hand we proceeded to carry out nonenzymatic primer extension experiments to quantitatively interrogate the effect of 2-thio substitution on RNA template-copying rates (Fig. 2). We used a primer/template complex composed of a DNA primer strand ending in a 3'-NH<sub>2</sub>-G and a complementary RNA template strand containing a 5'-C<sub>2</sub>A<sub>4</sub> overhang. pK<sub>a</sub> of the protonated 3'-amine of 3'-amino-2',3'-dideoxy-2-thio-thymidine is 7.5 (see the ESI†), similar to that reported for 3'-amino-2',3'-dideoxy-T (7.7).<sup>15</sup> 3'-NH<sub>2</sub>-2-MeImpddNs tends to undergo intramolecular cyclization due to the proximity of the primary 3'-amine group to the phosphorus electrophile.<sup>8</sup> Because the half-life (*t*<sub>1/2</sub>) of 3'-NH<sub>2</sub>-2-MeImpddT is 1.2 h<sup>8</sup> and that of 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T is 1.3 h (see the ESI† for details) under optimized primer extension conditions [100 mM 1-(2-hydroxyethyl)-imidazole, pH 7.5, 4 °C], we tracked primer extension only up to a maximum of 1 h. We determined observed rate constants *k*<sub>obs</sub> for the first step of the primer extension by following the loss of unreacted primer over time (Fig. 2).

At a 10 mM initial concentration of 3'-NH<sub>2</sub>-2-MeImpddT (Fig. 2, left), *k*<sub>obs</sub> of primer extension was 0.42 h<sup>-1</sup> (Table 1, entry 1). Notably, 2-thio modification led to about a 5-fold rate enhancement (Fig. 2, right), such that *k*<sub>obs</sub> for 10 mM 3'-NH<sub>2</sub>-2-MeImpdds<sup>2</sup>T was 1.92 h<sup>-1</sup> (Table 1, entry 2). This increase likely results from the additional stabilization induced into the primer/template duplex afforded by the formation of a s<sup>2</sup>T:A base pair compared to a canonical T:A base pair,<sup>16</sup> as well as the more 3'-endo-like sugar puckering of 2-thio-nucleotide, which is the favoured sugar conformation in nonenzymatic primer extension reactions.<sup>17</sup> The *k*<sub>obs</sub> values for reactions containing the activated ribonucleotides<sup>18</sup> 2-MeImpU, 2-MeImps<sup>2</sup>U and its ribo-T analog 2-MeImps<sup>2</sup>T were all



**Fig. 2** Kinetics of copying a  $r(A)_4(C)_2$  template with  $3'-\text{NH}_2\text{-2-MeImpddT}$  (left) and  $3'-\text{NH}_2\text{-2-MeImpdds}^2\text{T}$  (right), in the presence of  $100\text{ mM}$  1-(2-hydroxyethyl)-imidazole, at  $\text{pH } 7.5$  and  $4\text{ }^\circ\text{C}$ . Reactions were initiated by addition of monomers and monitored by gel electrophoresis. The triangle indicates the primer + 4 product. (bottom) Natural log of the fraction of the unreacted primer plotted against incubation time. Errors were based on two experiments. Primer strand (DNA,  $0.2\text{ }\mu\text{M}$ ): 5'-(FAM)-AGC-GTG-ACT-GAC-TGG-( $\text{NH}_2$ )-3', obtained enzymatically in *ca.* 85% purity based on LC-HRMS (see the ESI†). Primer concentration was corrected for unreactive oligonucleotide impurity. Template strand (RNA,  $1\text{ }\mu\text{M}$ ): 5'-CCAAAA-CCA-GUC-AGU-CAC-GCU-3' RNA.

**Table 1** Reaction kinetics measured for  $10\text{ mM}$  T/U monomers at  $4\text{ }^\circ\text{C}$

Entry	Template	Monomer	$k_{\text{obs}}\text{ (h}^{-1}\text{)}$	Relative $k_{\text{obs}}$
1 <sup>a</sup>	$r(A)_4(C)_2$	$3'-\text{NH}_2\text{-2-MeImpddT}$	0.42 (1)	7
2 <sup>a</sup>	$r(A)_4(C)_2$	$3'-\text{NH}_2\text{-2-MeImpdds}^2\text{T}$	1.92 (2)	30
3 <sup>b</sup>	$r(A)_6$	2-MeImpU	ND	—
4 <sup>b</sup>	$r(A)_6$	2-MeImps <sup>2</sup> U	0.064 (1)	1
5 <sup>b</sup>	$r(A)_6$	2-MeImps <sup>2</sup> T	0.22 (6)	3

<sup>a</sup> In the presence of  $100\text{ mM}$  1-(2-hydroxyethyl)imidazole. <sup>b</sup> Data obtained from ref. 18. Reactions performed with  $200\text{ mM}$   $\text{MgCl}_2$  at  $\text{pH } 7.0$ .

lower than the values for the activated  $3'$ -amino nucleotides described above (Table 1), even though these ribonucleotide polymerizations were assayed in the presence of  $200\text{ mM}$   $\text{Mg}^{2+}$  to optimize the reactivity. The rate enhancement observed for 2-MeImps<sup>2</sup>T *vs.* 2-MeImps<sup>2</sup>U suggests that methylation at the 5-position of 2-thiouracil leads to stronger monomer–primer stacking. Additionally, primer extension reactions with  $3'-\text{NH}_2\text{-2-MeImpdds}^2\text{T}$  are 10-fold faster than with the corresponding ribonucleotide, 2-MeImps<sup>2</sup>T, presumably due to the greater nucleophilicity of the  $3'$ -amine. Remarkably, combining the effect of the  $3'$ -amine and the 5-methyl groups results in an *ca.* 30-fold

(Table 1, entries 2 *vs.* 4) faster reaction. Further physical and kinetic characterization will be required to distinguish the contributions of enhanced monomer binding *vs.* enhanced monomer reactivity for these observations.

In conclusion, we have developed a scalable synthesis of a 2-thio modified thymidine monomer,  $3'-\text{NH}_2\text{-2-MeImpdds}^2\text{T}$ . Our synthetic route provided this highly reactive nucleotide in sufficient amounts to perform quantitative measurements of nonenzymatic RNA template-copying rates for the first time. Our results show that  $3'-\text{NH}_2\text{-2-MeImpdds}^2\text{T}$  can polymerize on a DNA/RNA primer/template complex significantly faster than any other U or T monomer that has been reported thus far.

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